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The relationship between corrosion and biodegradation of bio- and petroleum-based fuels was evaluated using aerobic seawater, fuel and unprotected carbon steel coupons under stagnant conditions to simulate a potential fuel storage condition. Aerobic respiration and corrosion reactions consumed oxygen in the incubations in a short time. The transient oxygen influenced the microbial biodegradation of all fuels and resulted in a suite of characteristic metabolites, including catechols. The corrosion was believed to be the result of biogenic sulfide production and in all cases, the black corrosion products contained chlorine and sulfur (presumed chloride and sulfide) in addition to iron. There were few differences in electrochemically measured corrosion rates in incubations amended with any of the fuels or their blends. Clone library analysis demonstrated higher proportions of Firmicutes, Deltaproteobacteria (primarily sulfate-reducing bacteria), Chloroflexi, and Lentisphaerae in incubations exposed to fuels than the original seawater. Relative proportions of sequences affiliated with these bacterial groups varied with fuel. Methanogen sequences similar to those of *Methanolobus* were also found in multiple incubations. Despite the dominance of characteristically anaerobic taxa, sequences coding for an alkane monooxygenase from marine hydrocarbon-degrading genera and aerobically produced intermediates were observed, indicative that organisms with this metabolic potential were active at some point during the incubation. Aerobic oxidation of fuel components resulted in the formation of a series of intermediates that could be used by anaerobic seawater microbial communities to support metabolism, sulfide production, and carbon steel corrosion.

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Oxygen Effects on Biodegradation of Fuels in a Corroding Environment

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Abstract

Experiments were designed to evaluate biodegradation of plant- and petroleum-based fuels and blends exposed to seawater under anaerobic conditions. The experimental set-up included natural aerobic seawater, fuel and unprotected carbon steel coupons, simulating a potential fuel storage situation. Corrosion was due to microbiologically produced sulfides reacting with carbon steel. There were few differences between electrochemically measured corrosion rates between plant-based, petroleum fuels or their mixtures. Transient oxygen influenced both metabolic degradation pathways and resulting metabolites. Detection of catechols suggested that initial exposure to oxygen resulted in their formation. Transient exposure to oxygen resulted in higher proportions of Firmicutes, Deltaproteobacteria (primarily sulfate-reducing bacteria), Chloroflexi, and Lentisphaerae in seawaters exposed to fuels than the original seawater. Relative proportions of sequences affiliated with these bacterial groups varied with fuel. Methanogen sequences similar to those of *Methanolobus* species were found in multiple incubations. Despite the dominance of characteristically anaerobic taxa, sequences coding for an aerobic hydrocarbon-degrading enzyme (alkane monooxygenase) were obtained following PCR amplification. The alkane monooxygenase and 16S rRNA clone libraries from seawater contained sequences with high similarity to marine hydrocarbon-degrading genera, mainly Gammaproteobacteria, suggesting that seawater was the source of these organisms and that they survived the anaerobic incubations.

Keywords: anaerobic biodegradation; transient oxygen; alternative fuel; corrosion

1. Introduction

Reducing dependence on fossil fuels requires not only increased energy efficiency and conservation, but also greater reliance on carbon neutral biofuels. First generation biofuels, a mixture of monoalkyl esters of long chain fatty acids (Ng et al. 2010), rapidly biodegrade under anaerobic conditions (Aktas et al. 2010). More recently, hydroprocessed (HP) bio-based lipids from renewable plant stocks (e.g. camelina and algae) are being considered as candidate alternative fuels. The HP fuels have chemical and physical characteristics that allow them to be readily blended with conventional petroleum products (Kalnes et al., 2007). However, the biological stability of HP fuels needs to be critically assessed under realistic storage and use conditions. Fuels are often stored for months in uncoated carbon steel tanks and exposed to varying amounts of fresh water and oxygen. Fuels can also come in direct contact with marine waters in ships equipped with seawater-compensated ballast tanks. In these systems, seawater is used to compensate for volume and weight loss as the ship's engines consume fuel.

Past experiments have demonstrated the following: 1) dissolved oxygen (DO) in stagnant natural seawater (8 ppm) exposed to corroding carbon steel, with or without fuel will be depleted to the detection limit (100 ppb) with 48 hours, 2) most solid surfaces in contact with natural seawater are anaerobic because of microbial respiration in a biofilm, 3) sulfate-reducing bacteria (SRB) dominate the microflora in marine biofilms and anaerobic seawaters and 4) sulfide influenced corrosion is the major mechanism for deterioration of metals used to transport and store fuels in contact with seawater. Experiments described in this paper were designed to examine the relationship between biodegradability of petroleum- (petro-) and plant (bio-) based fuels and biodegradation of carbon steel in fuel/natural seawater exposures. Observations were coupled with weight loss and instantaneous corrosion rate measurements for carbon steel coupons. Localized corrosion was evaluated on carbon-steel coupons using electrochemistry and

electron microscopy. Alterations in microbial community composition were monitored before and after a 90 d incubation period and metabolite profiling was used to deduce biodegradation pathways. Dissolved sulfide was measured in the seawater at the conclusion of the experiment.

2. Materials and Methods.

2.1 Fuel/Seawater Incubations

Fuels (petro-F76, petro-JP5, algal-F76, camelina-JP5, ultra low sulfur diesel (ULSD) and soy-based biodiesel) were obtained from Naval Fuels and Lubes Cross Functional Team, NAVAIR. Four neat fuels were used in these experiments including petroleumF76, petroleumJP5, camelina-JP5 and ULSD. Neat algal-F76 was not included due to limited quantity available. Three blended fuels were also used including 80:20 mix ULSD and soy biodiesel (BD 20), 50:50 mix of algal- and petro-F76, and 50:50 mix camelina- and petro-JP5. Coastal seawater was collected from Key West, FL (Lee et al. 2007) and used as an inoculum and growth medium. Seven chambers were constructed to contain seawater and one of the fuels. The chambers were cylindrical (35.5 cm diameter and 27.9 cm height) and constructed from heavy gauge, chemical resistant, opaque black plastic. Seawater (4.5 L) and fuel (3 L) were sealed in plastic chambers as previously described (Lee et al., 2004; Lee et al., 2005). An eighth chamber was used as a control with 4.5 L of seawater with no fuel addition. The chambers were placed in an anaerobic glove box (0.01% CO_2, 10% H_2, bal. N_2) where CO_2 concentration was chosen to maintain a seawater pH of between 7.8 and 8.2 (Lee et al. 2010a). Temperature was maintained at 23°C. No attempt was made to sterilize glassware, coupons, wires, or plastic mounts prior to introduction of fuel and water.

2.3 Corrosion

Carbon steel (UNS C10200) coupons (0.20% C, 0.47% Mn, 0.012% P, 0.013% Si, bal. Fe) (1.58 cm dia. X 0.16 cm) were individually mounted in epoxy (Epothin™, Buehler Ltd., Lake Bluff, IL) that established an exposed area of 2 cm^2. Prior to mounting, an insulated wire was attached to the backside of each coupon with conductive epoxy and carbon tape to achieve electrical connection. The as-received mill scale finish was left intact. Prior to exposure, mounted coupons were rinsed sequentially with acetone, ethanol and dried with N_2.

Coupons were arranged vertically within the chambers so that triplicate coupons were exposed to two conditions: 1) seawater immersion and 2) fuel immersion, where the lighter fuel formed a separate layer on top of the seawater. Polarization resistance (R_p) (ohms-cm^2) was monitored daily for seawater immersed coupons using the linear polarization technique (Scully, 2000) with a scan rate of 0.6 V/hr according to ASTM G59 (2003). The inverse of polarization resistance ($1/R_p$) (ohms-cm^{-2}) is proportional to the instantaneous corrosion rate. Silver/silver chloride (Ag/AgCl) electrodes and platinum/niobium (Pt/Nb) mesh were used as reference and counter electrodes, respectively. At the end of the incubation period, seawater H_2S concentration was measured as previously described (Lee et al., 2010).

Coupon weight loss was determined after acid cleaning for 10 minutes in room temperature 1:1 hydrochloric acid (sp gr 1.19):deionized water with 3.5 g L^{-1} hexamethylene tetramine (ASTM G1 2003). Coupons were imaged (before and after acid cleaning) in an environmental scanning electron microscope (ESEM) at an accelerating voltage of 20 kev.

Energy dispersive spectroscopy (EDS) was used to evaluate the chemical composition of corrosion products.

2.3 Metabolite profiling

To assess putative polar metabolites and other components that partitioned into the water phase, fuels were mixed with equal volumes of seawater, stirred overnight, and the resulting aqueous phase was acidified (10N HCl; pH $\leq$ 2) prior to an ethyl acetate extraction. Extracts were concentrated, derivatized and analyzed by gas chromatography-mass spectrometry (GC-MS) as previously described (Aktas et al., 2010). This baseline was then compared to the fuel-seawater mixtures that were incubated for 90 days and analyzed in an identical manner. In addition, fuel layers were tested for metabolites before and after the incubations. All identifications were made by comparison of the GC retention times and mass spectral fragmentation profiles of commercial standards (Sigma-Aldrich, St Louis, Mo) that were similarly analyzed, or by comparison with the NIST Mass Spectral Library, Version 2.0a.

2.4 Microbial community analysis

2.4.1 Extraction of DNA

At the start of the experiment, one liter of seawater was filtered through a Nalgene filter unit (PES membrane, 0.2 micron pore size). DNA was extracted from the filter using the MegaPrep UltraClean Soil DNA Extraction Kit (MO BIO Laboratories) and concentrated by ethanol precipitation according to manufacturer's instructions. Seawater samples from the fuel incubations were collected at the end of incubation and identically filtered. Filters were placed in sterile 50 mL centrifuge tubes, immediately frozen, and shipped on ice to Norman, OK, where the filters were stored at -75°C prior to DNA extraction. PowerSoil™ DNA Isolation Kit (MO BIO Laboratories) was used to extract the DNA from the filters.

2.4.2 Q-PCR estimation of bacterial 16S rRNA and *mcrA* gene copy numbers

Numbers of bacterial 16S rRNA gene copies and of *mcrA* (subunit *a* of methyl-S-CoM methylreductase, indicative of methanogens) were estimated using q-PCR. The PCR reactions for bacterial 16S rRNA genes contained 2xSyber mix (Applied Biosystems, Carlsbad, CA), 0.5 mM Betaine, 0.075 µl of 100 µM 8F primer and 0.0375 µl of 100 µM 338R primer (Stevenson et al. 2011) with the balance of the 30 µL reaction mix provided by RT-PCR grade water (Applied Biosystems, Carlsbad, CA) and template DNA. The PCR reactions for *mcrA* contained 2xSyber mix (Applied Biosystems, Carlsbad, CA), 0.5 mM betaine, 0.3 µl of 100 µM ml as primer and 0.3 µl of 100 µM mcrA-rev primer (Steinberg and Regan, 2008) with the balance of the 20 µL reaction mix provided by RT-PCR grade water and template DNA. Thermal cycling was performed by the StepOnePlusTM Real-Time PCR System (Applied Biosystems, Carlsbad, CA, StepOne Software v2.1). Cycling conditions for 16S rRNA gene sequence copies were: 95°C for 10 minutes followed by 40 cycles of 96°C for 30 seconds, 55°C for 45 seconds, and 72°C for 45 seconds (Stevenson et al. 2011). Cycling conditions for the *mcrA* gene sequence copies were: 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds, 55° for 45 seconds, 72°C for 30 seconds, and image capture at 83°C, followed by a final extension at 72°C for 7 minutes (Steinberg and Regan, 2009). Samples were run in triplicate, with standard curves provided by serial dilutions of a plasmid containing a single copy of a bacterial 16S rRNA sequence (for 16S rRNA q-PCR) or a single copy of a methanogen *mcrA* gene sequence (for *mcrA* q-PCR). The

DNA concentration of the plasmid standards was quantified with the Qubit® 2.0 Fluorometer and Qubit® dsDNA HS Assay kit (Invitrogen Corporation Life Technologies, Carlsbad, CA).

2.4.3 Microbial community profiling

Partial bacterial 16S rRNA gene fragments (approximately 1300 bp) were amplified from seawater DNA with the primers 27F and 1391R using the primer sequences and protocol for PCR amplification specified in the Department of Energy Joint Genome Institute (JGI) protocol for RNA Library Creation (http://my.jgi.doe.gov/general/protocols/SOP_16S18S_rRNA_PCR_Library). Partial bacterial 16S rRNA gene fragments (approximately 900 bp) were amplified from the DNA extracted from the incubations with the primers 27F and 907R (Muyzer et al., 1998). Partial archaeal 16S rRNA gene fragments (approximately 600 bp) were amplified using the primers ARC333 and 958R (Reysenbach and Pace, 1995), as described in Gieg et al. (2008). Degenerate primers targeting *alkB* (a catalytic subunit of alkane monooxygenase, Kloos et al., 2006) were used to create libraries of aerobic degradative pathway functional genes. Clone libraries for Key West seawater and for the incubations were created using the TOPO TA Cloning Kit (Invitrogen Corp., Carlsbad, CA). The sequencing was performed on an ABI model 3730 capillary sequencer using the M13 flanking regions as sequencing primer sites (Microgen: The Laboratory for Genomics and Bioinformatics, Oklahoma City, OK). Additional sequences were obtained by direct amplification with M13 primers of the insert from transformed cells and purification of the PCR product with EXO-SAP-IT (USB). Sequencing directly from transformed cells was performed at the Oklahoma Medical Research Sequencing Facility (Oklahoma City, OK) using flanking M13 vector sequences. Sequencher (Gene Codes Corp. Ann Arbor, MI) was used to examine the chromatograms and create consensus sequences from the forward and reverse reads.

2.4.4 Analysis of DNA sequences

Consensus sequences from the 16S rRNA libraries were analyzed using the mothur software package (Schloss et al., 2009). Sequences were clustered into operational taxonomic units (OTUs) at the 3% dissimilarity level (e.g. 97% similarity) and classified at the 60% level using the rRNA Classifier (Ribosomal Database Project, Wang et al., 2007). BLASTN searches (Altschul et al., 1990) were used to compare representative sequences from dominant OTUs to the GeneBank database. Distance matrices (DeSantis et al. 2006) were created from the *alkB* libraries and used by the DOTUR program (Schloss and Handelsman 2005) to identify groups of sequences sharing the 97% level of nucleotide sequence similarity. BLASTN searches (Altschul et al. 1990) were used to compare a representative sequence from each *alkB* OTU to the GeneBank database. Representative sequence data reported from the 16S and *alkB* libraries were deposited in the GenBank database under accession numbers *JN986847-JN986872.*

3. Results

3.1 Partitioning of fuel components to seawater

Fuel components partitioned to seawater from fuel blends qualitatively reflected both the petro- and bio-based parent fuels. For example, a comparison of petroleum and algal-based F76 and their 50:50 mix is illustrated in Figure 1. The comparison showed a qualitatively comparable suite of *n*-alkanes (C_{10} to C_{23}) partitioned to seawater, regardless of the parent fuel (Figure 1). Compared to the algal-F76, the petro-F76 exhibited a more complex gas chromatographic profile

and more components resolved at both relatively early and late retention times. These compounds could also be detected when the seawater was exposed to the F76 blended mixture. Resolvable components from both parent fuels in the fuel blends were observed for all fuels (data not shown).

All fuels and blends contained straight chain alkanes (C_{11} to C_{15}) that partitioned to the seawater phase. Some fuels, including petro-F76, ULSD and their blends contributed *n*-alkanes up to C_{24}. Qualitatively, a complex suite of iso-alkanes of varying carbon chain lengths (C_9 to C_{19}) also partitioned to seawater from all fuel types. Longer chain branched alkanes were more pronounced in F76 and ULSD fuels than in JP5 fuels. Partitioned constituents from biodiesel were fatty acid methyl esters (FAME) with chain lengths that ranged from C_5 and C_{20}. Similar components also partitioned to seawater from BD20.

A comparison of the chromatographic profiles after the 90-day exposure indicated that all *n*-alkanes [C_{10} to C_{16}] in petro- and camelina-JP5 were removed (Figure 2). Similarly, whether the hydrocarbons were contributed by petro- or camelina-JP5, the same pattern of *n*-alkane removal was observed in the 50:50 JP5 mix. The number of components that resolved in the GC analysis was particularly striking when the initial and final spectra for camelina-JP5 were compared. The initial camelina-JP5 had a complex chromatographic profile of compounds that partitioned to the seawater phase. However, after 3 months incubation with seawater, most of these components were at or below detection limits. Moreover, several new peaks were observed in all JP5-related GC spectra after incubation that were not detected in either the original fuels or seawater (Figure 2).

In contrast, a different pattern of hydrocarbon loss was evident with the F76 fuels (Figure 3). Removal of *n*-alkanes removal was far less prominent in the petroleumF76 incubations, but loss of other fuel components was evident. When the petroleum F76 was mixed equally with algal-F76 and incubated with seawater, the preferential removal of low and medium molecular weight *n*-alkanes (C_{10}-C_{15}) was apparent. Ratios of the integrated peak areas changed in the chromatographic profiles for lower (and not the higher) molecular weight *n*-alkanes, suggesting that simple dilution cannot account for the removal of these fuel components.

There was no dramatic change between the initial and the final gas chromatographic profiles of the ULSD incubations in terms of *n*-alkane content. However, new peaks that were not present at the start of the experiment were detected after the incubation (Figure 4). The n-alkanes were partially removed in BD20 incubations. More obvious however, was a substantial reduction of fatty acid methyl ester features in the final relative to the initial profile. This is particularly evident with the prominent unsaturated C_{18} methyl ester peak (labeled with a black dot) (Figure 4).

The removal of branched alkanes was also evident in the chromatographic profile. Branched alkanes comprise a relatively small portion of the chromatographic profile, and while resolved, the iso-alkane peaks are small relative to the *n*-alkanes. Nevertheless, the differences in the branched alkane content between initial and final time points paralleled that observed for *n*-alkanes. More specifically, branched alkanes were almost completely depleted in the seawaters exposed to JP5 fuels and the 50:50 mix F76, whereas they were only partially depleted in petroleumF76, ULSD and BD 20 incubations.

3.2 Corrosion

Instantaneous corrosion rates ($1/R_p$ [$ohms^{-1}cm^{-2}$]) for carbon steel immersed in seawater with petroleumF76 addition are shown in Figure 5. Over the first 7 days, corrosion rate decreased by an order-of-magnitude. On day 10 a sulfide odor was detected and corrosion rate began to increase. By day-30, a 2 order-of-magnitude increase in corrosion rate had occurred compared with the minimum day-7 value. For the remainder of the 90-day exposure, the corrosion rate remained at the elevated level. This basic curve shape was observed for all exposures regardless of fuel type addition. One exception was the BD20 exposure where the minimum corrosion rate after 7 days was an order-of-magnitude lower (10^{-6} ohm^{-1} cm^{-2}) than that measured for the other exposures.

At the conclusion of the experiment all chambers had a "rotten egg" sulfide smell. Dark corrosion products were observed on all coupons in proximity to seawater whether fully immersed or at the seawater/fuel interface. Coupons in the fuel layer exhibited regions of localized corrosion (pitting) surrounded by unattacked metal (Figure 6) whereas coupons immersed in seawater were consistently pitted. Corrosion products in both the water and fuel phases contained chloride and sulfur (EDS spectra not shown). Dissolved sulfide was detected in all seawaters with the highest concentration (150 ppm) in the seawater with 50:50 JP-5 addition.

Table 1 lists the weight loss (g) for carbon steel after the 90-day exposure and acid cleaning. Weight loss in the fuel and seawater layers was similar. Exceptions included camelina-JP5 where significantly more weight loss occurred in the seawater and ULSD and BD20 where weight loss in the seawater was greatly suppressed. The lowest amount of metal loss occurred in the seawater with BD20 addition.

3.3 Metabolite profiling

Fuel layers were analyzed by GC-MS before and after the incubations. No metabolites were detected. At the end of the incubation, several putative metabolites were detected in all incubations (Table 2). All incubations except those amended with petroleumF76, contained catechol and/or a variety of alkylated catechols. Phenol, cresol and benzoate were other common metabolites in the incubations that could be formed under both aerobic and anaerobic conditions. Small molecular weight alcohols and fatty acids were also detected, the latter being more pronounced in the BD20 incubations.

3.4 Microbial community analysis

3.4.1 Q-PCR estimation of bacterial 16S rRNA and *mcrA* gene copy numbers

The number of bacterial 16S rRNA gene sequence copies in the seawater sample was approximately 10^6/mL (Table 3). Incubations amended with fuels had considerably lower numbers of bacterial 16S rRNA copies (Table 3). Copy numbers were lowest in the 50:50 mix F76 and ULSD incubations (123 copies/mL and 265 copies/mL, respectively), higher in the petroleum and camelina-JP5 and petroleumF76 incubations (647 copies/mL, 871 copies/mL, and $3.7x10^3$ copies/mL) and approximately 10^5/mL in the 50:50 mix JP5 and BD20 incubations.

The number of methanogens was estimated by Q-PCR of a portion of a gene sequence coding for an enzyme essential for methanogenesis, methyl-S-CoM methylreductase (subunit α, *mcrA*). Methanogens were scarce in seawater relative to the estimated numbers of bacteria

(approx. 1/1000 that of estimated bacterial 16S rRNA gene sequences). However, the ratio of 16S rRNA to *mcrA* gene copies was less than 1 for BD20 incubations. This suggests that methanogenesis may have been an important process. This ratio was almost equal to 1 in the petroleumF76 incubations and approximately 20:1 in the camelina-JP5 and 50:50 mix JP5 incubations. The *mcrA* genes were not detected by this method in the petroleumJP5, 50:50 mix F76, and ULSD incubations. The 50:50 mix F76 and ULSD incubations also had the lowest estimated number of bacterial 16S rRNA gene sequence copy numbers.

3.4.2 Microbial community profiling

Sequences from the different fuel incubations showed distinctively different community profiles (Figure 9). The 73 sequences in the seawater 16S rRNA gene sequence library formed 45 operational taxonomic units (OTUs) at the 97% level of similarity. The three most abundant groups in seawater were affiliated with Alphaproteobacteria (34.2%), Bacteriodetes (30.1%), and Gammaproteobacteria (17.8%) (Supplementary Table 1). In many cases, the OTUs showed highest similarity to cloned sequences of samples from coral reefs (Gammaproteobacteria: Chromatiales, AB294951; and Bacteriodetes: Sphingobacteriales, FJ202681), the Atlantic Ocean (Alphaproteobacteria, Rhodobacteraceae, EU795149), and Arabian Sea (Firmicutes, AY907773).

In contrast, approximately half the sequences in the 16S rRNA gene sequence libraries (total: 309 sequences, 90 OTUs) were affiliated with Firmicutes or Deltaproteobacteria. The distribution of sequences affiliated with different groups varied with the fuel (Supplementary Table 1): the incubation containing ULSD was dominated by Firmicutes, Delta- and Gammaproteobacteria, and Alphaproteobacteria, while the BD20 incubation was strongly dominated by Firmicutes (75%). The incubations amended with petroleumJP5 and F76 or camelina-JP5 had the highest percentage of Deltaproteobacterial sequences (38-60% of total), while Chloroflexi, though found only in the petroleumF76, 50:50 mix JP5, and 50:50 mix F76-containing incubations made up a substantial fraction at 30-40%. The cells with 50:50 mix JP5 and 50:50 mix F76 were noteworthy for having a high proportion of Lentisphaerae (22% and 35%, respectively).

The distribution of sequences grouped into OTUs at the 97% level of similarity was examined for the most abundant OTUs to determine whether the most abundant OTUs were unique to particular fuels or were more widely distributed. The 14 most abundant OTUs (e.g. those with 5 or more sequences per OTU, approximately 61% of total # sequences) consisted of 4 OTUs affiliated with Firmicutes, 4 with Deltaproteobacteria, 3 with Chloroflexi, 1 with Planctomycetes, 1 with Sphingobacteriales, and 1 with Lentisphaerae (Supplementary Table 2). Overall, 3 of the 4 Firmicute OTUs, and the Sphingobacteriales occurred in 4 or more fuel incubations. The Deltaproteobacteria, Chloroflexi, Planctomycetes, and Lentisphaera OTUs were not as widely distributed in the incubations as the Firmicutes, although several occurred in 3 different incubations. Some of the OTUs appeared to be specific to certain fuel incubations: the most abundant Deltaproteobacteria OTU (47 sequences, 98% similarity to AB470955, *Desulfovibrio* sp. r02), occurred in petroleumJP5 (24 sequences) and camelina-JP5 (21 sequences, 2 sequences in 50:50 mix JP5) while the next most abundant Deltaprotobacteria OTU (13 sequences, 99% similarity to AF418172, *Desulfovibrio profundus*) was found exclusively in petroleumF76. All three of the most abundant Chloroflexi OTUs appeared in the same three fuel incubations, e.g. petroleumF76, 50:50 mix JP5, and 50:50 mix F76.

3.4.3 Archaeal community profiling

Good PCR amplification for archaeal 16S rRNA sequences was obtained from seawater, BD20, petroleumF76, and 50:50 mix JP5 incubations (Figure 8). Sequences of methanogens affiliated with *Methanolobus* species were the only archaeal 16S rRNA sequences found in the seawater and incubations with BD20 or petroleumF76. Incubations with 50:50 mix JP5 was most diverse, some *Methanolobus*-affiliated and other methanogen sequences, but also representatives of Thermoprotei, Thermoplasmata, and several archaeal sequences similar to uncultured clones that were not classifiable at the 60% level of similarity (RDP Classifier). The dominant *Methanolobus* 16S rRNA sequences were highly similar to those of *Methanolobus* sp. HigM (AB370247) or *Methanolobus profundi* (AB370245), a methylotrophic methanogen isolated from a natural gas field (Mochimaru et al., 2009).

3.4.4 Alkane monooxygenase sequences

Sequences identified as coding for a portion of an enzyme that initiates aerobic hydrocarbon degradation (*alkB,* alkane monooxygenase) were obtained following PCR-amplification with specific primers from all the fuel incubations with the exception of camelina-JP5. The cloned sequences were grouped into OTUs with 97% nucleotide sequence similarity (Figure 9, representing 29 sequences). Each fuel incubation contained from 2 to 4 different OTUs. Sequences were affiliated with *alkB* gene sequences derived from well-known alkane-degrading marine bacteria such as *Alcanivorax, Kordiimonas, Oleiphilus, Gaetbuilibacter,* and *Marinobacter* (gammaproteobacteria), and *Silicibacter* (alphaproteobacteria).

4. Discussion

Analysis of fuel constituents that partitioned to seawater suggested that many of the water-soluble fuel components were biodegraded. The removal of almost all alkanes from the incubations of bio-based fuels (and their blends) suggests that these fuels were somewhat more amenable to decay than their petroleum-based fuel counterparts. However, JP5 was an exception in this regard since all *n*-alkane peaks were removed from the gas chromatographic profile. The highest measured dissolved sulfide (150 ppm) was measured in the seawater of the tank containing the 50:50 mix of JP 5 fuels. The most abundant Deltaproteobacteria OTU (47 sequences, 98% similarity to AB470955, *Desulfovibrio* sp. r02), occurred in petroleumJP5 (24 sequences) and camelina-JP5 (21 sequences, 2 sequences in 50:50 mix JP5) This was probably due in part to the fact that petroleum JP5 consisted of mostly low to medium molecular weight *n*-alkanes (up to C_{15}) while the other two fuels contained these and higher molecular weight paraffins up to 24 carbons in chain length. This result might be explained by the preferential utilization of smaller *n*-alkanes by the seawater microflora.

Similarly n-alkanes were removed from the BD20 incubation. FAME components of biodiesel are known to be more labile than hydrocarbons to anaerobic biodegradation (Aktas, et al, 2010), so the preferential loss of these peaks from the chromatographic profiles confirms previous findings.

At the end of 90 days a variety of putative metabolites were observed in the incubations supporting the contention that hydrocarbon removal was due at least in part to biodegradation by seawater microorganisms. Benzoate, cresols and alkanoic acids observed in the incubations could conceivably be the result of either aerobic or anaerobic metabolic processes. However, the

formation of a series of catechols is particularly diagnostic. The appearance of catechols could only result from the initial aerobic biodegradation of aromatic compounds (Mallick et al., 2011). This suggests that the incubations were exposed to oxygen at some point during the 90 d incubation. Subsequent aerobic microbial metabolism of catechols requires molecular oxygen. The persistence of the catechols suggests that oxygen was either unavailable for subsequent microbial metabolism or that the anaerobic biodegradation of the substrate was slow relative to the metabolism of other potential substrates. These metaboites are also good indicators that oxygen was present at some point. However, it is quite likely that oxygen reserves were exhausted and essentially unavailable for the subsequent aerobic microbial metabolism of the resulting catechols. The aerobic microorganisms that are capable of catechol metabolism typically elaborate dioxygenases that require molecular oxygen as a co-substrate for ring cleavage reactions (ref). Indeed, previous experiments using the same experimental system confirmed that oxygen, while initially present at low levels in seawater, was depleted within ≤48 h due to consumption by corrosion reactions and aerobic heterotrophic respiration (Lee et al., 2004, 2005). The transient nature of the oxygen exposure was enough to allow the seawater microflora to partially oxidize a variety of aromatic hydrocarbons. Upon oxygen depletion, the resulting oxidation products as well as the parent hydrocarbons were subject to anaerobic decomposition processes. Fatty acids could be intermediates formed during the aerobic or anaerobic microbial metabolism of alkanes or from the hydrolysis of the FAME components and subsequent metabolism via beta-oxidation.

Supporting the presence of oxygen in the incubations at some point, were sequences coding for a portion of an enzyme that initiates aerobic hydrocarbon degradation (*alkB,* alkane monooxygenase). These sequences originated from the same groups of marine gammaproteobacteria and alphaproteobacteria that were found in the seawater sample, specifically those with high similarity to *Gaetbulibacter, Oleiphilus, Alcanivorax, Kordiimonas*, and *Silicibacter*, suggesting seawater as the source of bacteria capable of aerobic hydrocarbon degradation. The aforementioned alkane-degrading gamma- and alphaproteobacteria, many of which specialize in hydrocarbon-degradation ("Obligate hydrocarbonoclastic bacteria", Yakimov et al. 2007), have been found repeatedly in marine systems. Sequences related to oil-degrading Oceanospirillales (gammaproteobacteria) increased dramatically in response to the Deepwater Horizon blowout (Hazen et al., 2010). Thus, the ubiquity and rapid response shown by this group of bacteria suggest that seawater is a source of aerobic hydrocarbon-degrading bacteria that are also likely capable of attacking HP alternative fuels.

The shift in bulk conditions from aerobic to anaerobic is inevitable when the limited concentration of oxygen was consumed in the incubations. There was approximately 30 mM of sulfate available in Key West seawater that was reduced to sulfide via anaerobic microorganisms. (The total amount of sulfide produced does not reflect the amount of sulfate reduced since the sulfide forms insoluble complexes with metals in the incubations). Initially, the anaerobes would not be dominant members of oxygenated seawater communities and in fact are difficult to even detect. However, such communities can change rapidly in response to the prevailing environmental conditions, in this case, low or no oxygen and the availability of labile organic carbon. In contrast to the aerobic marine microflora found in the seawater sample, bacterial communities from fuel-amended incubations had much higher proportions of typically anaerobic groups such as clostridial Firmicutes and Deltaproteobacteria. Although the 50:50 fuel blends were chemically a good reflection of both the bio- and petroleum based fuels, the bacterial

profiles were quite different. Particularly noteworthy were the lower proportion of Deltaproteobacteria and the appearance of Lentisphaerae in the 50:50 blends compared to the corresponding single-fuel incubations. The phylum-level group Lentisphaerae is considered rare and estimated to be less than 1% of the community obtained from Oregon coastal seawater (Cho et al., 2004). Two properties make *Lentisphaera* an intriguing subject for future biofilm formation and biocorrosion studies, i) the two cultivated strains are noted for the production of transparent exopolymeric material, and ii) the genome of the type strain was found to possess 267 putative sulfatases (Thrash et al., 2010). The prospective role of these organisms in the blended fuel metabolism as a reason for their enrichment as well as their role in corrosion is unknown.

Our calculations (not shown) suggest that it should easily be possible to deplete the seawater of sulfate, assuming that hydrocarbon removal was due to anaerobic biodegradation processes. Under these circumstances, methanogenesis would likely become an increasingly important terminal electron accepting process. While methane formation was not specifically monitored in our incubations, the presence of 16S rRNA sequences of methanogens and Q-PCR estimates of *mcrA* genes in the incubations amended with F76, biodiesel, and 50:50 JP5 mix suggests that methanogenesis was likely a prominent process in at least some of the fuel-amended incubations. As oxygen sensitive metabolic process, the enrichment of methanogens as well as the increase in the detection of a functional gene attest to the very strongly anaerobic and reducing conditions present in our incubations, despite the fact that the seawater was initially aerobic.

The relationship between biodegradability of fuels exposed to seawater and biodegradation of carbon steel is extremely complex. In the experiments described in this paper, all tanks received the same amount of seawater and therefore the same amount of sulfate and chloride and the same inoculum. The fuels differed in the details of their chemistry and the constituents that were partitioned to the seawater. Growth of the microorganisms depended on the biodegradability of those constituents. Pathways for the biodegradation, however, were influenced by transient oxygen. Corroding carbon steel influenced biodegradation by contributing to the removal of oxygen and the removal of sulfides produced by SRB. In a closed system, as described in this work, dissolved sulfides can be toxic to SRB. Corroding carbon steel produced Fe^{+2} that could precipitate S^{-2}, permitting more growth SRB, fuel degradation and corrosion. The experiments described in this paper cannot valuate all of the possible feedback reactions in this complex medium.

5. Conclusions

Observations related to fuel biodegradation and carbon steel corrosion in fuel/seawater combinations for petroleum-based fuels were similar to those for petroleum-based fuels with the same basic chemistry. The metabolic fate of the water-soluble fuel components from all fuels was influenced by the presence of transient oxygen in seawater. The presence of diagnostic catechols confirmed that aerobic microorganisms in the seawater initiated the aerobic metabolism of the parent aromatic hydrocarbons. Microbial community profiling confirmed that aerobic microorganisms persisted in a anaerobic (100 ppb) environment for three months.

6. Acknowledgements

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Table 1. Weight loss (g) of carbon steel after acid cleaning.

	Seawater	Fuel
Seawater Only	0.151	-
ULSD	0.074	0.120
BD20	0.034	0.117
JP5 Petro	0.101	0.156
JP5 Camelina	0.126	0.093
JP5 50/50	0.088	0.074
F76 Petro	0.137	0.142
F76 50/50	0.088	0.074

Table 2. Metabolites in the incubations at the end of 90 days

	Cam[a] JP5	Petro JP5	JP5 Mix[b]	Petro F76	F76 Mix[b]	ULSD	BD 20[c]
Alcohols							
Cyclohexanol	+	+	+	+	-	+	-
Cyclohexen-1-ol	+	-	-	-	-	-	-
Hexanol	-	-	-	-	+	-	-
Octanol	-	-	+	-	-	-	-
Monoaromatics							
Benzoate	+	+	+	-	-	+	-
Phenol	+	+	+	+	+	-	+
Cresol	-	+	+	+	+	+	-
Catechol	-	+	+	-	+	-	+
3,5-di-tert-butylcatechol	+	+	+	-	-	+	-
2,6-di-tert-butylbenzene-1,4-diol	-	+	+	-	-	-	-
Dimethylphenols	-	+	+	+	+	-	-
2 or 3-hydroxyacetophenone	-	+	+	+	+	-	-
Alkanoic acids							
C3	-	-	-	-	-	-	+
C5	-	-	+	+	-	-	+
C6	-	+	+	+	+	+	+
C8	-	-	-	-	+	-	-
C9	+	-	-	-	-	-	-
C10	-	-	-	+	-	-	-
C12	-	-	-	+	+	-	
Octanedioic acid	-	-	-	-	-	-	+
Nonadioic acid	-	-	-	-	-	-	+

[a] camelina
[b] 50:50 mixture of petro- and bio-based fuels.
[c] 80:20 mixture of ULSD:Biodiesel fuels.

Table 3. Number of bacterial 16S rRNA and mcrA gene copies estimated by Q-PCR

Sample type[a]	# bac 16S genes/mL[b]	# mcrA genes/mL[c]	# bac 16S genes/ # mcrA genes
Seawater	1.22×10^6	2.48×10^3	4.92×10^2
PetroleumJP5	8.71×10^2	BDL[d]	-
Camelina-JP5	6.47×10^2	31.4	20.60
50:50 mix JP5	2.01×10^5	1.32×10^4	15.22
PetroleumF76	3.70×10^3	3.51×10^3	1.05
50:50 mix F76	1.23×10^2	BDL	-
ULSD	2.65×10^2	BDL	-
BD20	2.25×10^5	5.13×10^5	0.44

[a] Fuel type incubated with seawater under transient oxygen conditions.
[b] Estimated number of bacterial 16S rRNA gene copies per mL sample
[c] Estimated number of *mcrA* (subunit *a* of methyl-S-CoM methylreductase, indicative of methanogens) gene copies per mL sample.
[d] BDL: below the detection limit, approximately 10 copies/mL

Figure Legends

Figure 1. Gas chromatographic profiles of the fuel components that partitioned to the aqueous phase for petroleum and algal-F76 and their 50:50 blend. The alkanes are labeled with their corresponding carbon chain lengths. The specific peaks are marked with dashed lines to make the comparison between profiles of different fuels easier.

Figure 2. Gas chromatographic profiles of the fuel components that partitioned to the aqueous phase for petroleum and camelina-JP5 and their 50:50 blend before and after the incubations. The alkanes are labeled with their corresponding carbon chain lengths. The specific peaks are marked with dashed lines to make the comparison between profiles of different fuels easier.

Figure 3. Gas chromatographic profiles of the fuel components that partitioned to the aqueous phase for petroleum and algal-F76 and their 50:50 blend before and after the incubations. The alkanes are labeled with their corresponding carbon chain lengths. The specific peaks are marked with dashed lines to make the comparison between profiles of different fuels easier.

Figure 4. Gas chromatographic profiles of the fuel components that partitioned to the aqueous phase for ULSD and BD20 blend before and after the incubations. The alkanes are labeled with their corresponding carbon chain lengths. The specific peaks are marked with dashed lines to make the comparison between profiles of different fuels easier.

Figure 5. Averaged logarithmic R_p^{-1} instantaneous corrosion rates ($ohms^{-1}$ cm^{-2}) of carbon steel immersed in seawater under petro F76 as a function of exposure time (days). The basic shape of this curve was common among all exposures regardless of fuel type.

Figure 6. Localized corrosion of carbon steel after 90 days exposure in petroleumJP5 layer of a seawater/fuel mixture.

Figure 7. Relative abundance of bacterial Phyla (class level for Proteobacteria) from 16S rRNA gene libraries in the pristine seawater and fuel incubations.

Figure 8. Relative abundance of dominant archaeal operational taxonomic units (OTUs) and groups from 16S rRNA gene libraries. Dominant OTUs (97% level of similarity) are "Thermoprotei1", "Methanolobus1", "Methanolobus2", and "Methanolobus3". More than one OTU is represented by the groups "other Thermoprotei", "other Methanomicrobia", "Thermoplasmata", and "unclassified". The sample type is indicated by the label on the x axis.

Figure 9. Phylogenetic relationships of *alkB* sequences with respect to related sequences. The tree is constructed from approximately 548 bp using the neighbor-joining algorithm. One thousand bootstrap replications were performed; only values greater than 700 are shown. Bar represents 0.01 nucleotide substitutions per nucleotide. Sequences from this study representing OTUs at the 97% level of similarity are indicated by bold formatting.

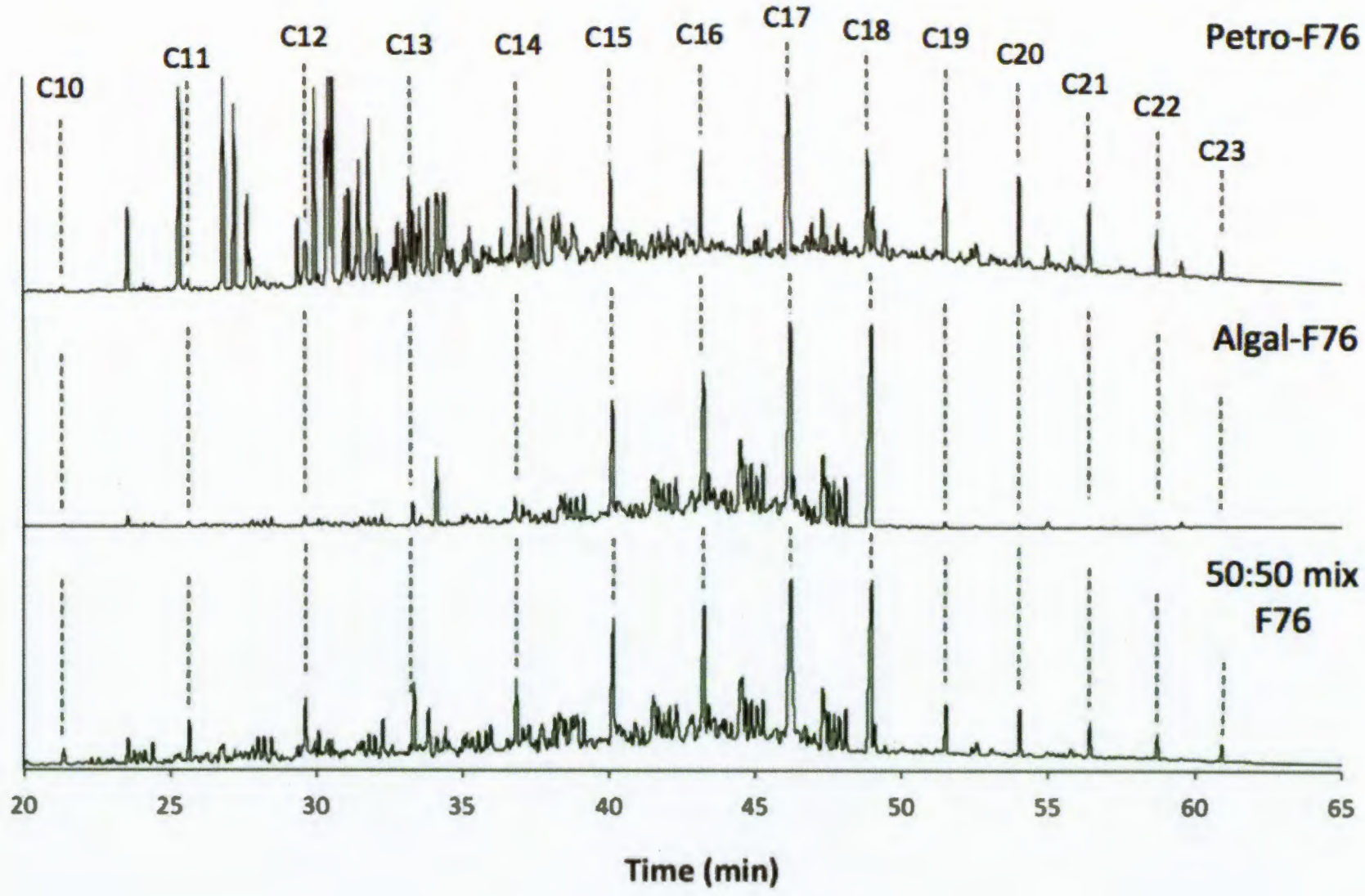
C10
C11
C12
C13
C14
C15
C16
C17
C18
C19
C20
C21
C22
C23
Petro-F76
Algal-F76
50:50 mix
F76
20
25
30
35
40
45
50
55
60
65
Time (min)

Figure 1

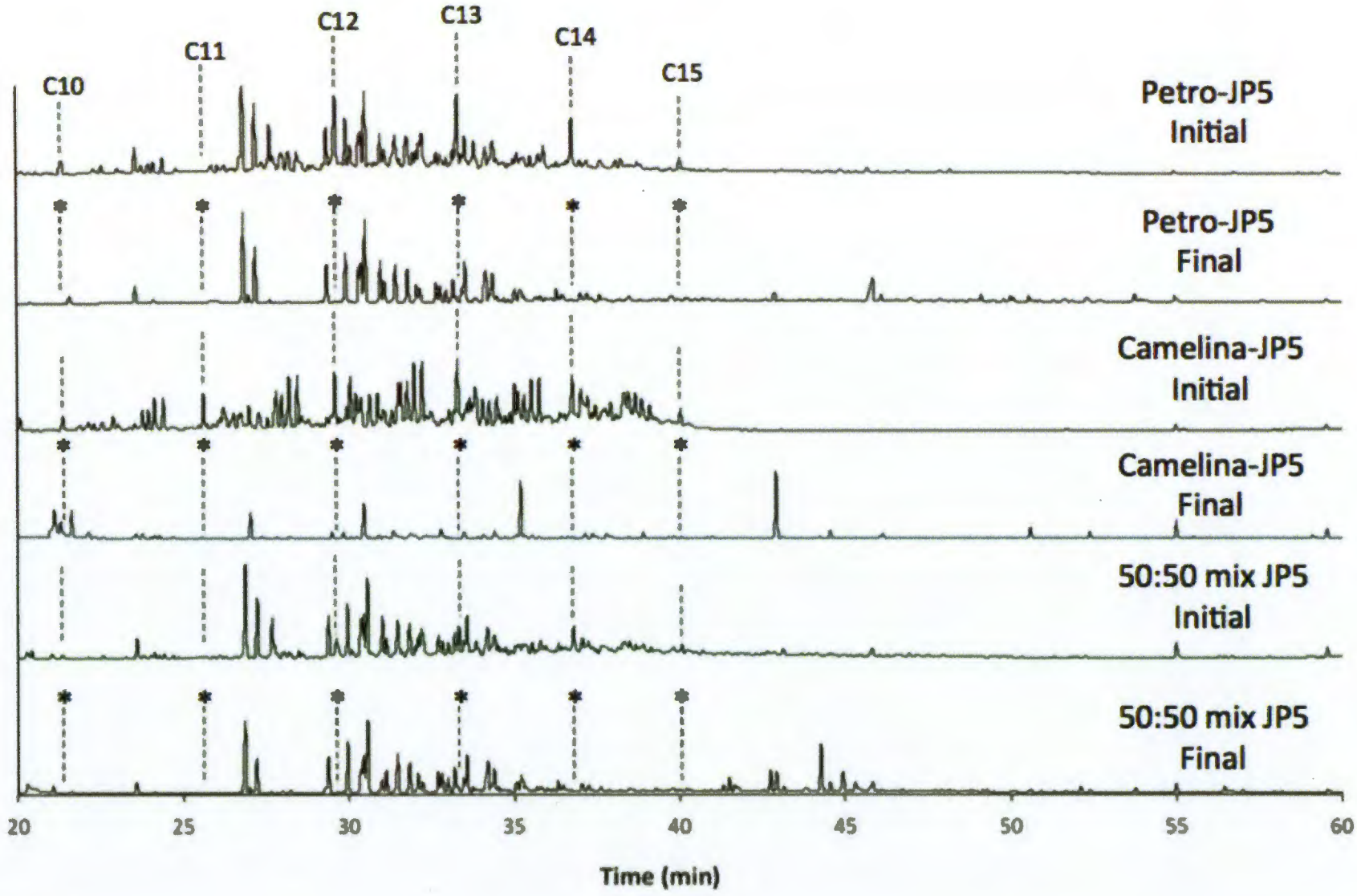
C10
C11
C12
C13
C14
C15
Petro-JP5 Initial
Petro-JP5 Final
Camelina-JP5 Initial
Camelina-JP5 Final
50:50 mix JP5 Initial
50:50 mix JP5 Final
20
25
30
35
40
45
50
55
60
Time (min)

Figure 2

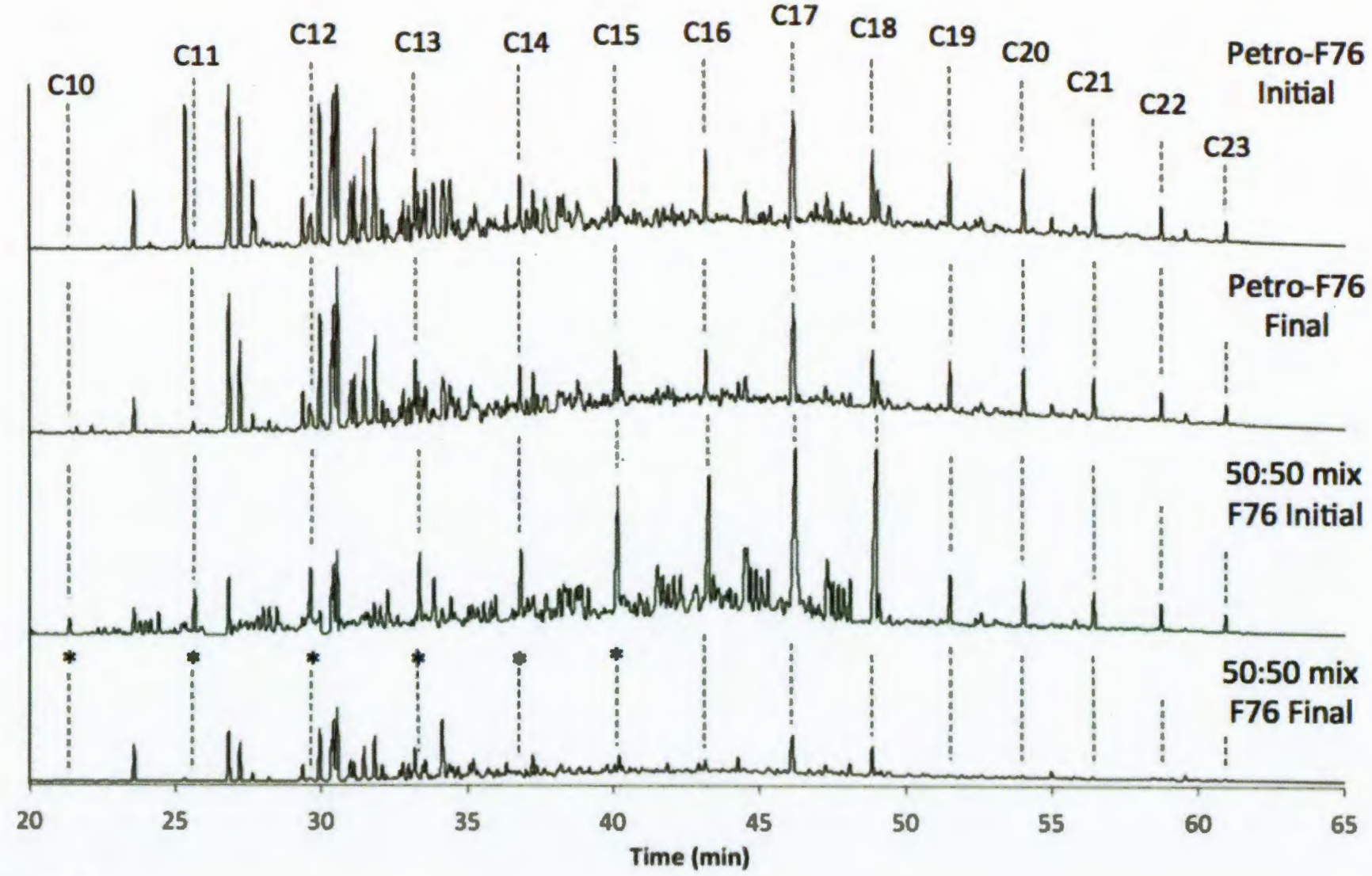
C10
C11
C12
C13
C14
C15
C16
C17
C18
C19
C20
C21
C22
C23
Petro-F76 Initial
Petro-F76 Final
50:50 mix F76 Initial
50:50 mix F76 Final
20
25
30
35
40
45
50
55
60
65
Time (min)

Figure 3

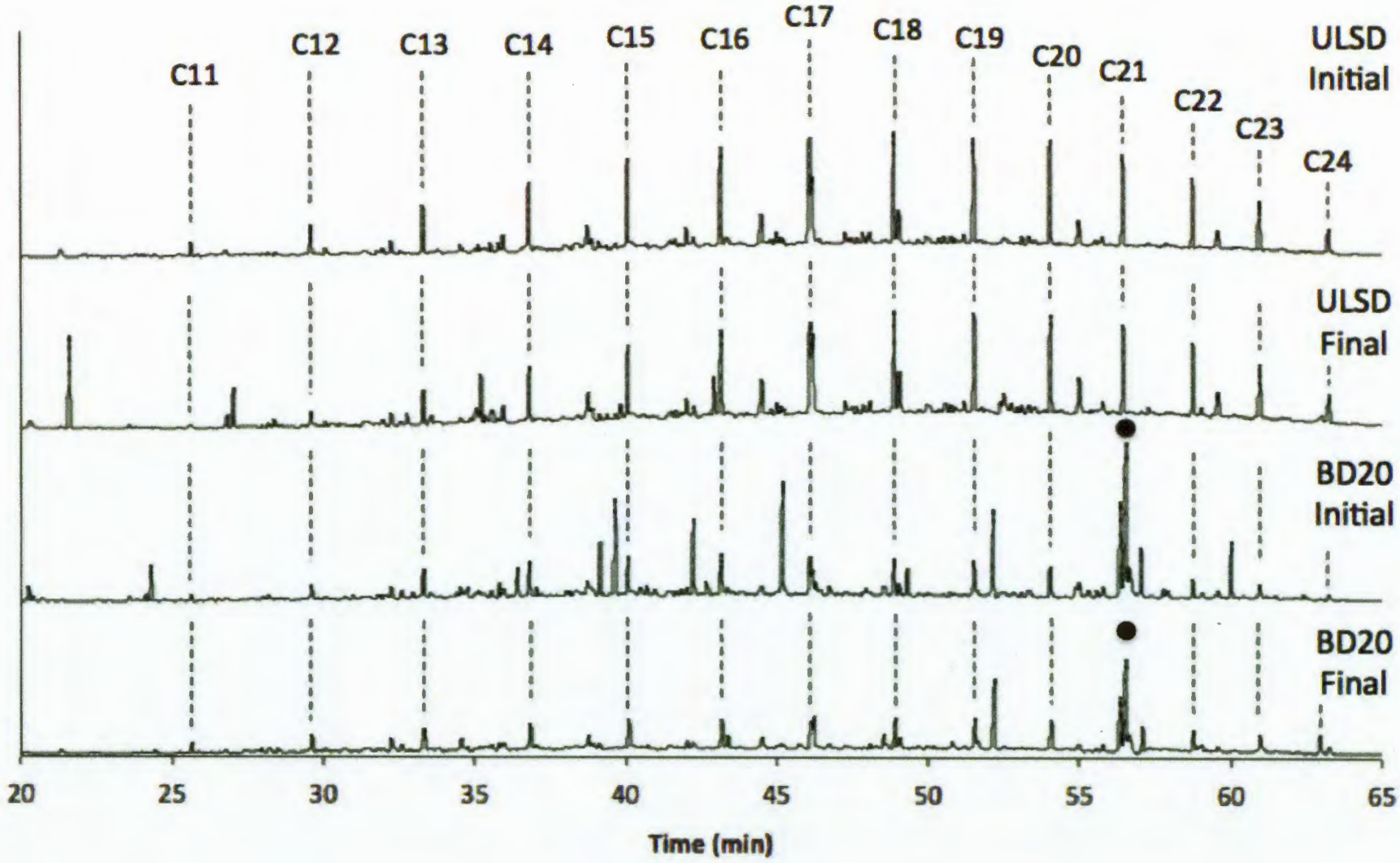
C11
C12
C13
C14
C15
C16
C17
C18
C19
C20
C21
C22
C23
C24
ULSD Initial
ULSD Final
BD20 Initial
BD20 Final
20
25
30
35
40
45
50
55
60
65
Time (min)

Figure 4

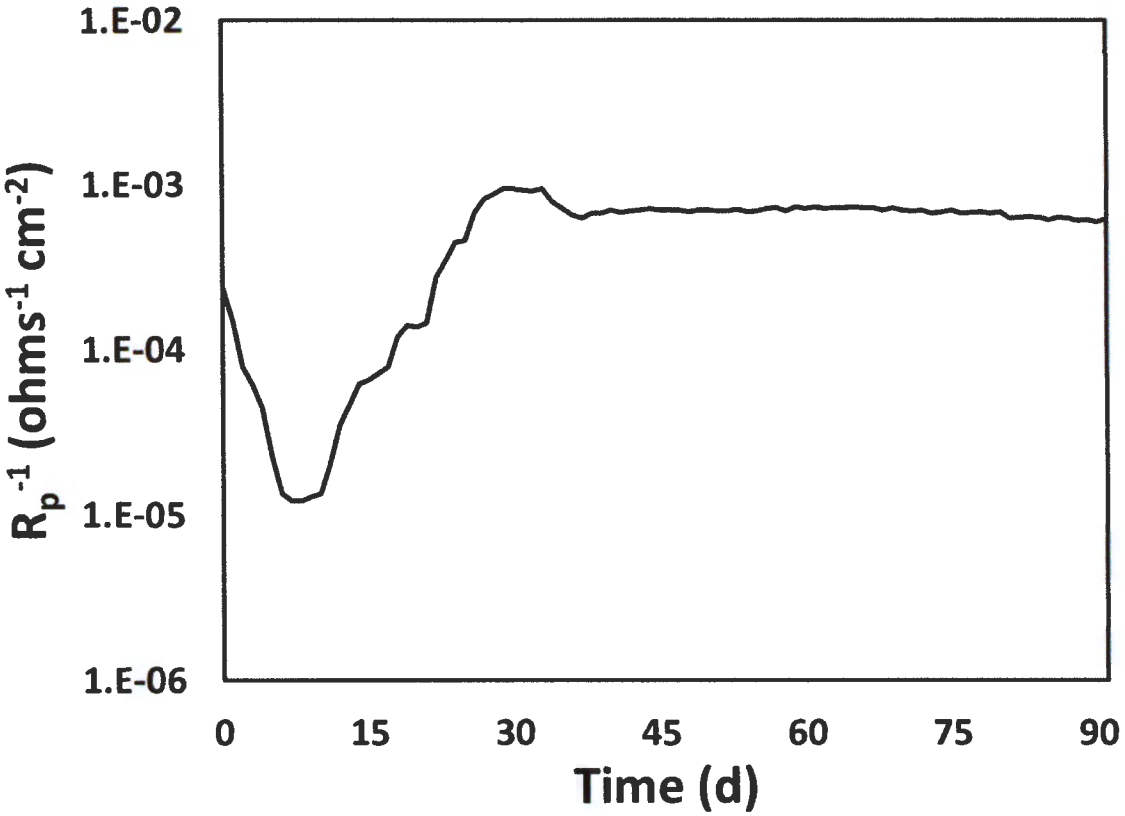

Figure 5.

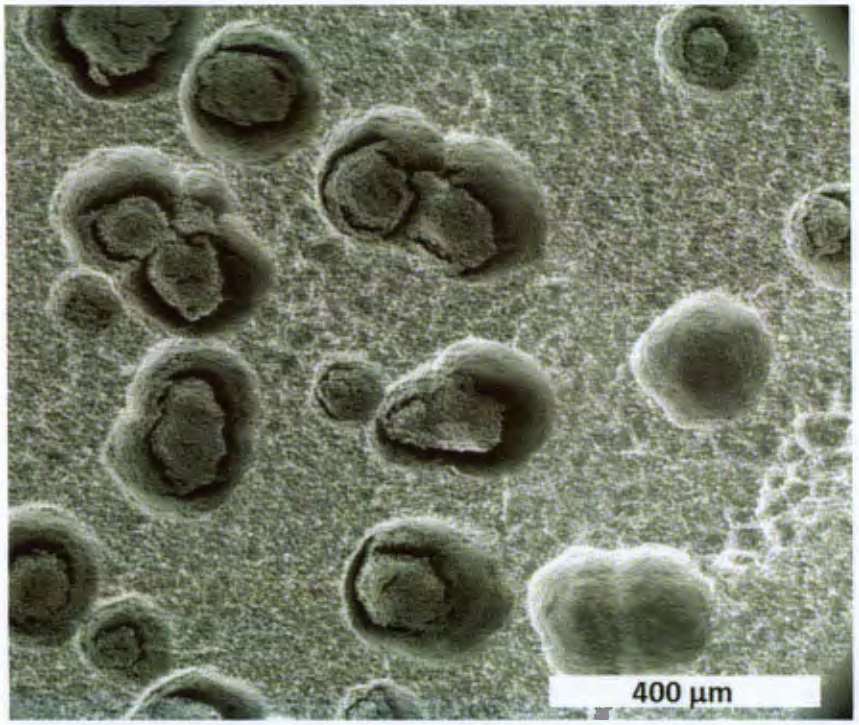

Figure 6.

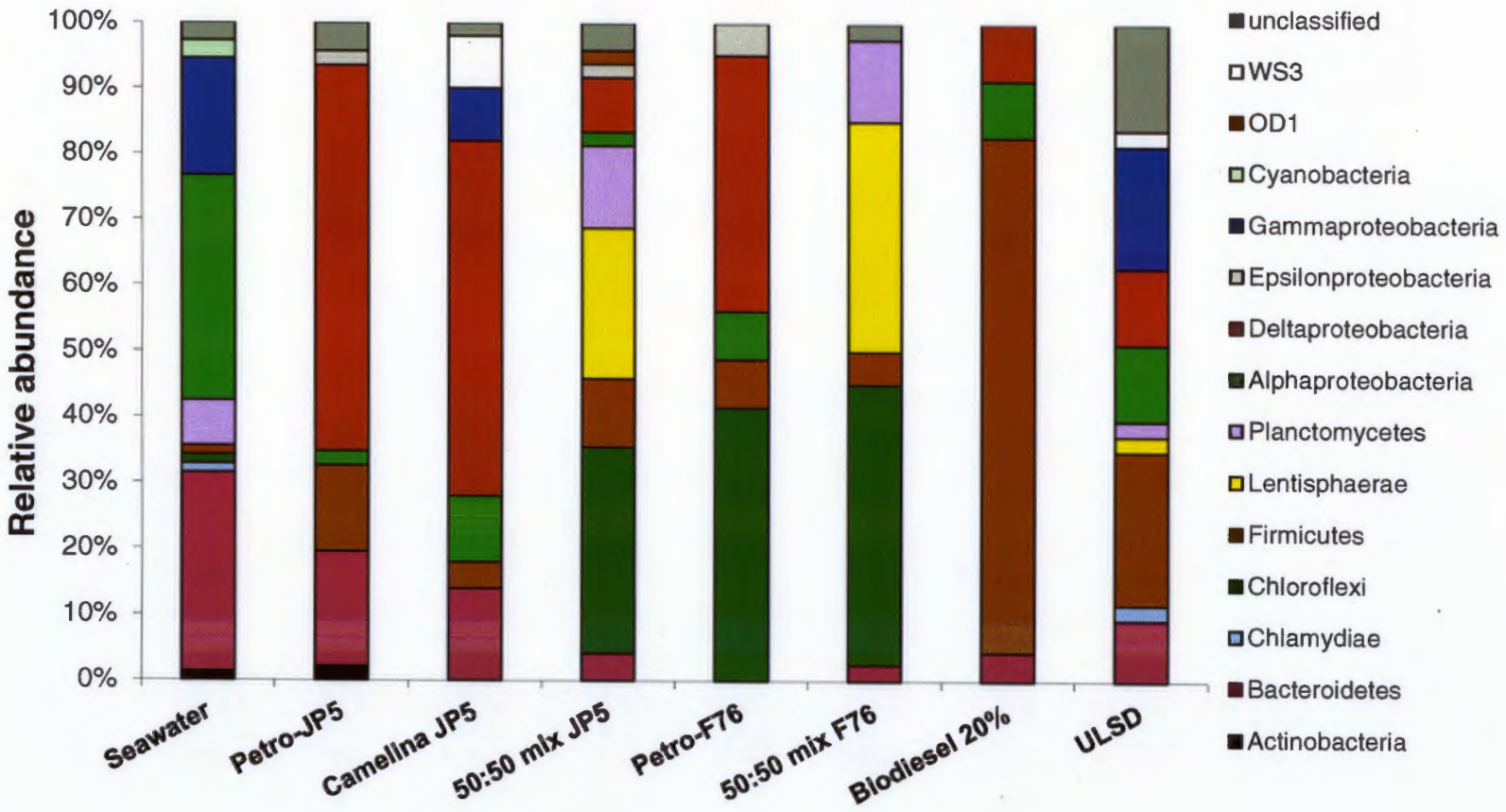
Relative abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Seawater
Petro-JP5
Camelina JP5
50:50 mix JP5
Petro-F76
50:50 mix F76
Biodiesel 20%
ULSD
unclassified
WS3
OD1
Cyanobacteria
Gammaproteobacteria
Epsilonproteobacteria
Deltaproteobacteria
Alphaproteobacteria
Planctomycetes
Lentisphaerae
Firmicutes
Chloroflexi
Chlamydiae
Bacteroidetes
Actinobacteria

Figure 7

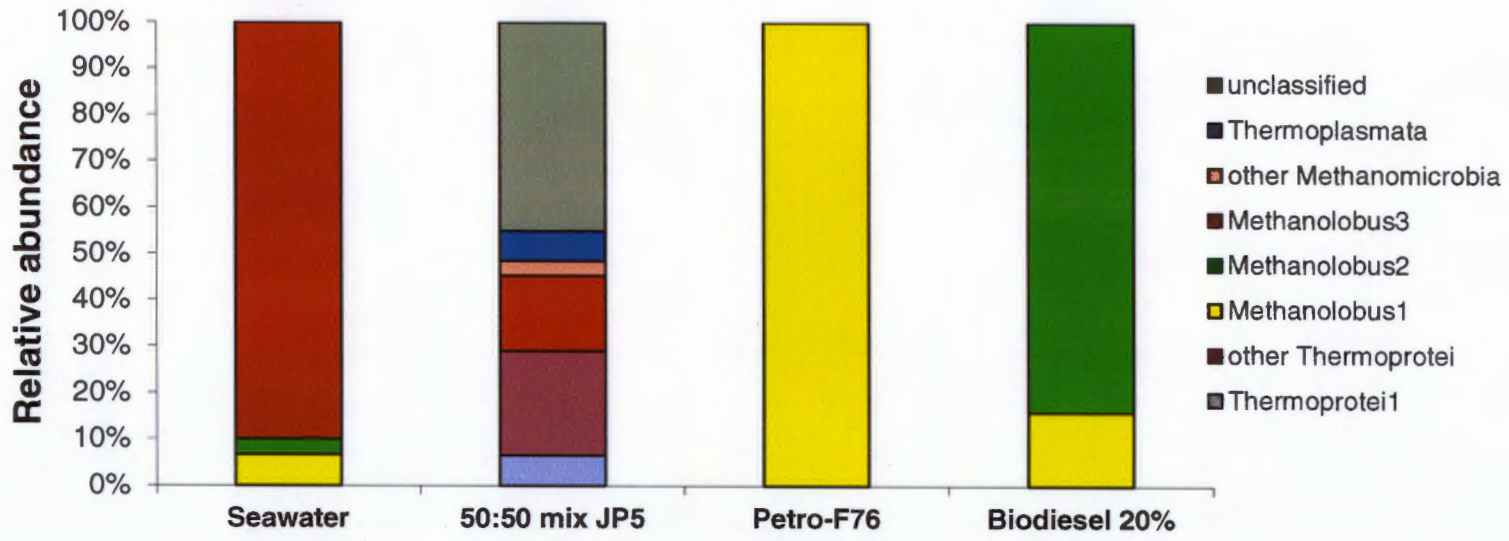
Relative abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Seawater
50:50 mix JP5
Petro-F76
Biodiesel 20%
unclassified
Thermoplasmata
other Methanomicrobia
Methanolobus3
Methanolobus2
Methanolobus1
other Thermoprotei
Thermoprotei1

Figure 8

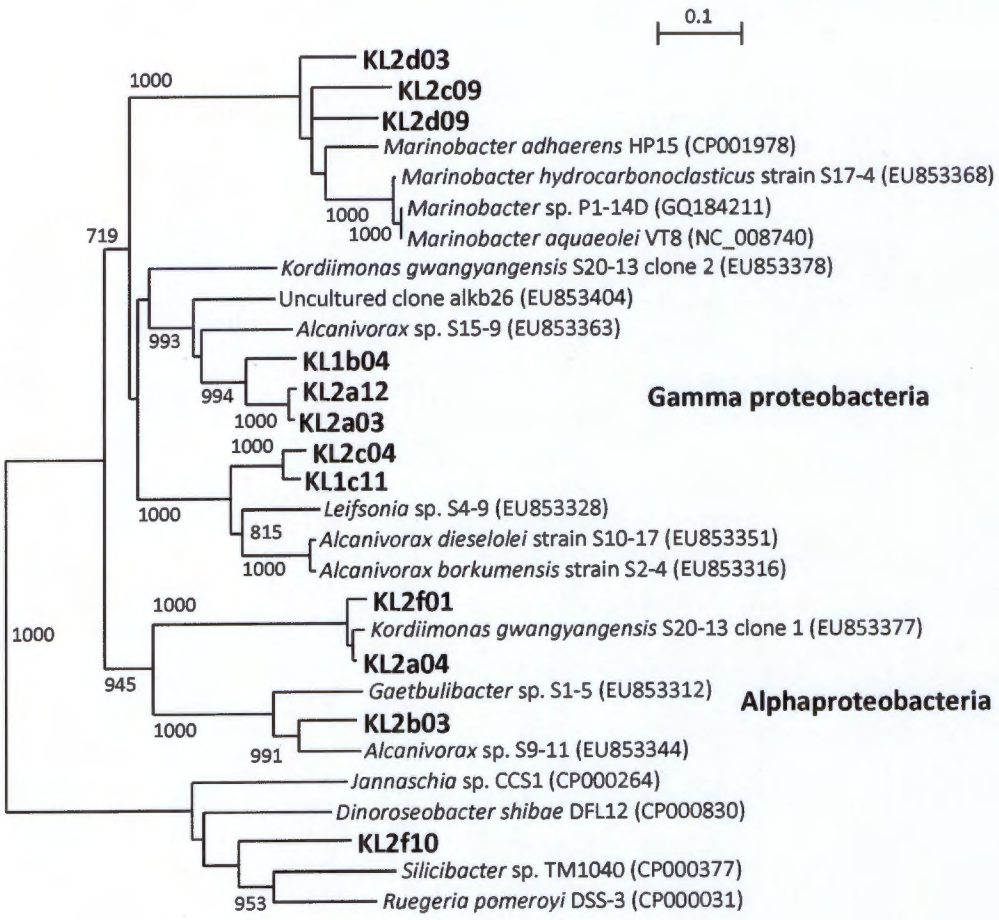
0.1
KL2d03
KL2c09
KL2d09
Marinobacter adhaerens HP15 (CP001978)
Marinobacter hydrocarbonoclasticus strain S17-4 (EU853368)
Marinobacter sp. P1-14D (GQ184211)
Marinobacter aquaeolei VT8 (NC_008740)
Kordiimonas gwangyangensis S20-13 clone 2 (EU853378)
Uncultured clone alkb26 (EU853404)
Alcanivorax sp. S15-9 (EU853363)
KL1b04
KL2a12
KL2a03
KL2c04
KL1c11
Leifsonia sp. S4-9 (EU853328)
Alcanivorax dieselolei strain S10-17 (EU853351)
Alcanivorax borkumensis strain S2-4 (EU853316)
KL2f01
Kordiimonas gwangyangensis S20-13 clone 1 (EU853377)
KL2a04
Gaetbulibacter sp. S1-5 (EU853312)
KL2b03
Alcanivorax sp. S9-11 (EU853344)
Jannaschia sp. CCS1 (CP000264)
Dinoroseobacter shibae DFL12 (CP000830)
KL2f10
Silicibacter sp. TM1040 (CP000377)
Ruegeria pomeroyi DSS-3 (CP000031)
1000
719
993
994
1000
1000
1000
1000
815
1000
1000
1000
945
1000
991
953
Gamma proteobacteria
Alphaproteobacteria

Figure 9

Supplementary information

Table S1. Distribution of phylogenetic affiliations of bacterial 16S rRNA gene sequence libraries

Affiliation	Seawater	Petro-JP5[1]	Camelina-JP5	50:50 mix JP5	Petro-F76	50:50 mix F76	BD20	ULSD
Actinobacteria	1.4%[2]	2.1%						
Chlamydiae	1.4%							2.3%
Lentisphaerae				22.4%		35.0%		2.3%
Verrucomicrobia							2.1%	2.3%
Planctomycetes	6.8%			10.2%		12.5%		2.3%
Bacteroidetes	30.1%	17.0%	14.0%	4.1%		2.5%	6.3%	9.1%
WS3			6.0%					2.3%
Firmicutes	2.7%	12.8%	4.0%	10.2%	7.1%	5.0%	75.0%	22.7%
Alphaproteobacteria	34.2%	2.1%	10.0%	2.0%	7.1%		8.3%	13.6%
Deltaproteobacteria		59.6%	54.0%	8.2%	38.1%		8.3%	18.2%
Epsilonproteobacteria		2.1%		2.0%	7.1%			
Gammaproteobacteria	17.8%		8.0%					18.2%
Chloroflexi	1.4%			32.7%	40.5%	42.5%		
OD1				2.0%				
Cyanobacteria	2.7%							
Unclassified	1.4%	4.3%	4.0%	6.1%		2.5%		6.8%

[1]Type of fuel incubated with seawater

[2] % total sequences classified (at least 60% confidence level, RDP Classifier) within the phylogenetic group listed under "Affiliation"

Table S2. Distribution of most abundant bacterial 16S rRNA gene sequence OTUs.

Affiliation	OTU representative	GenBank closest match	% similarity	Uncultured or isolate	Isolation source	# sequences in each fuel incubation							total # sequences
Deltaproteobacteria						Petro-JP5	Camelina-JP5	50:50 mix JP5	Petro-F76	50:50 mix F76	BD20	ULSD	
Desulfobulbaceae	N1a10	DQ112424	96	Uncultured	intertidal mudflatsediment	0	0	0	0	0	0	5	5
Desulfovibrio A	N1g06	AB470955	98	*Desulfovibrio* sp. r02	associated with coral	24	21	2	0	0	0	0	47
Desulfovibrio B	N2a04	AF418172	99	*Desulfovibrio profundus*	deep sediment layers in the Japan Sea	0	0	0	13	0	0	0	13
Desulfovibrio C	N4c03	AB470955	97	*Desulfovibrio* sp. r02	associated with coral	0	6	0	0	0	0	0	6
Firmicutes													
Clostridiales A	N1a11	AM176887	98	Uncultured	mangrove sediment.	2	1	0	0	0	2	1	6
Clostridiales B	N1b01	FJ671193	93	Uncultured	beef cattle feedlot	1	0	0	2	0	6	1	10
Clostriciales C	N1b04	GQ413702*	98	Uncultured	coral-associated	1	0	1	1	0	5	1	9
Lachnospiraceae	N1e01	AY216444	93	Uncultured	temperate estuarine mud	0	0	0	0	0	5	0	5
Chloroflexi													

Chloroflexi A	N2a03	GU180176	96	Uncultured	sediment and soil slurry	0	0	3	4	1	0	0	8
Chloroflexi B	N2a08	EU721826	97	Uncultured	Alaskan mesothermic petroleum reservoir	0	0	9	6	4	0	0	19
Chloroflexi C	N2e08	EF629773	99	Uncultured	sponge tissue	0	0	7	3	7	0	0	17
Other													
Lentisphaeria	N2e07	EU491113	91	Uncultured	seafloor lavas	0	0	4	0	13	0	0	17
Planctomycetales	N2e04	AM997823	94	Uncultured	deep-sea surface sediments, S. Atlantic Ocean	0	0	6	0	5	0	0	11
Sphngobacteriales	N1d05	FJ716343	93	Uncultured	Sawmill Sink water column, Bahamas	6	5	2	0	1	0	0	14